Cystic fibrosis transmembrane conductance regulator: the NBF1 + R (nucleotide-binding fold 1 and regulatory domain) segment acting alone catalyses a $\text{Co}^{2+}/\text{Mn}^{2+}/\text{Mg}^{2+}$ -ATPase activity markedly inhibited by both Cd^{2+} and the transition-state analogue orthovanadate

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Cystic fibrosis (CF) is caused by mutations in the gene encoding CFTR (cystic fibrosis transmembrane conductance regulator), a regulated anion channel and member of the ATP-binding-cassette transporter (ABC transporter) superfamily. Of CFTR's five domains, the first nucleotide-binding fold (NBF1) has been of greatest interest both because it is the major 'hotspot' for mutations that cause CF, and because it is connected to a unique regulatory domain (R). However, attempts have failed to obtain a catalytically active NBF1 + R protein in the absence of a fusion partner. Here, we report that such a protein can be obtained following its overexpression in bacteria. The pure NBF1 + R protein exhibits significant ATPase activity [catalytic-centre activity (turnover number) 6.7 min^{-1}] and an apparent affinity for ATP (K_m , $8.7 \mu\text{M}$) higher than reported previously for CFTR

or segments thereof. As predicted, the ATPase activity is inhibited by mutations in the Walker A motif. It is also inhibited by vanadate, a transition-state analogue. Surprisingly, however, the best divalent metal activator is Co^{2+} , followed by Mn^{2+} and Mg^{2+} . In contrast, Ca^{2+} is ineffective and Cd^{2+} is a potent inhibitor. These novel studies, while demonstrating clearly that CFTR's NBF1 + R segment can act independently as an active, vanadate-sensitive ATPase, also identify its unique cation activators and a new inhibitor, thus providing insight into the nature of its active site.

Key words: anion channel, ATPase, ATP-binding-cassette transporter (ABC transporter), cystic fibrosis transmembrane conductance regulator (CFTR), nucleotide domain.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease characterized by increased sweat chloride, pancreatic insufficiency and chronic lung infections, and frequently leads to an early death [1,2]. This debilitating disease is caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR), a regulated Cl^- channel [3] and member of the ATP-binding-cassette transporter (ABC transporter) superfamily [4]. CFTR consists of a single polypeptide chain of 1480 amino acids that fold into five distinct domains. These include two nucleotide-binding folds (NBF1 and NBF2), a regulatory domain (R) and two transmembrane domains (TM1 and TM2) [3]. Although mutations (> 800) that cause CF are distributed throughout CFTR [5], those that cause most forms of the disease reside in NBF1 and NBF2 with about 70% of all cases attributed to deletion of Phe-508 from NBF1 [1–3].

Generally, it is believed that within a functional CFTR molecule the three soluble domains (NBF1, NBF2 and R) form a complex (NBF1 + R/NBF2) in which each nucleotide domain is catalytic (i.e. exhibits ATPase activity), and interacts with the other via a co-operative process that is modulated by phosphorylation of the R domain (reviewed in [6–9]). The subsequent structural changes that ensue, and the order in which they occur,

are then believed to be important for regulating the activity of CFTR's Cl^- channel, comprising defined segments derived from TM1 and TM2 [8]. Evidence for this view has come from many careful studies including those designed to examine the effect of mutations within intact CFTR on channel function [6–8], studies focused on the isolation and characterization of individual domains [10–17] and, most recently, studies designed to reconstitute individual domains [14,18]. What is lacking is structural information at atomic resolution. This has not been forthcoming for either intact CFTR or any of its domains, although there have been two very recent notable successes for intact bacterial ABC transporters [19,20] and several for nucleotide domains isolated from bacterial and mammalian ABC transporters [21–24].

Unfortunately, efforts to acquire structural information at atomic resolution for either intact CFTR or its domains have been impeded by many difficulties in obtaining functional preparations in sufficient quantities. This in turn has slowed our understanding of the molecular and chemical basis of CFTR function, and therefore our understanding of those structural changes that occur in the many mutant forms that cause CF. Consequently, improved therapies based on rational drug design remain hopes for the future rather than realities of the present. One possible exception may involve the use in drug design of the

Abbreviations used: ABC transporter, ATP-binding-cassette transporter; Caps, 3-(cyclohexylamino)-1-propane sulphonic acid; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ht, histidine tag; IPTG, isopropyl β -D-thiogalactoside; NBF, nucleotide-binding fold; Ni-NTA, Ni^{2+} -nitrilotriacetic acid; R, regulatory domain; TBS, Tris-buffered saline; TM, transmembrane domain.

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recently solved NMR structure of a peptide segment representative of the Phe-508 region of NBF1 [25].

With the above in mind, one of our major objectives has been to obtain the NBF1 + R/NBF2 complex in functional form so that its structure and that of its major mutant forms that cause CF can be elucidated by crystallographic methods. Significantly, a similar structural goal was achieved previously for the mitochondrial ATP synthase's catalytic F_1 moiety [26,27], which consists of three major subunit types, two of which comprise nucleotide domains homologous to NBF1 and NBF2 of CFTR [28]. Although we have worked out procedures to purify both NBF1 + R and NBF2 as separate proteins and demonstrated via several methods that these proteins interact [14], we have not demonstrated that they can function separately as active ATPases or together as a single ATPase complex. The possibility that NBF1 + R and NBF2 each have the capacity to catalyse ATP hydrolysis has been suggested in brief reports [11,15] summarizing studies in which these proteins were first fused with other proteins.

In studies reported here we have focused on obtaining an NBF1 + R protein with the capacity to catalyse ATP hydrolysis in the absence of a fusion partner. We have not only accomplished this goal but have identified two new divalent metal ion activators of NBF1 + R's ATPase activity, a divalent metal ion inhibitor, and a transition-state-like inhibitor, thus obtaining valuable clues about the nature of its catalytic site and reaction mechanism.

MATERIALS AND METHODS

Materials

The following compounds or materials were purchased from Sigma: ATP, P_i , $CaCl_2$, $CdSO_4$, $CoCl_2$, $MgCl_2$, $MnCl_2$, 3-(cyclohexylamino)-1-propane sulphonic acid (Caps), dithiothreitol, glycerol, guanidine hydrochloride, imidazole, isopropyl β -D-thiogalactoside (IPTG), kanamycin, protein kinase A catalytic subunit from porcine heart, SDS, glutathione and Triton X-100. Sodium orthovanadate was also obtained from Sigma and pre-treated exactly as described previously [29] to minimize the presence of polymeric species. The Ni^{2+} -nitrilotriacetic acid (Ni-NTA) superflow material was from Qiagen, while Coomassie Blue stain, Silver Stain-Plus detection system, polyacrylamide, molecular-mass standards, PVDF membranes and mini-gel transfer system were from Bio-Rad. [γ - ^{32}P]ATP was from NEN Life Sciences, Malachite Green from Fluka, ammonium molybdate tetrahydrate from J. T. Baker and Centricon-100 filtration devices from Amicon. The donkey anti-mouse IgG conjugated to horseradish peroxidase and the enhanced chemiluminescence system were from Amersham Biosciences. Non-fat milk was obtained from Friendly Farms, and the anti-CFTR monoclonal antibody (MATG 1061) exhibiting specificity for NBF1 was a generous gift from Transgene (Strasbourg, France). Restriction enzymes were from New England BioLabs, the pET-28a(+) vector was from Novagen, and all primers were from the Johns Hopkins University, School of Medicine, Synthesis and Sequencing Facility housed in the Department of Biological Chemistry (Baltimore, MD, U.S.A.). Full-length human CFTR in the plasmid pBQ-4.7-CFTR was kindly provided by Dr Joanna Rommens (Hospital for Sick Children, Toronto, Canada). All other reagents were of the highest commercial purity available.

Construction of an expression vector for the NBF1 + R (Gly-404–Lys-830) region of human CFTR

The third base, A, within the restriction enzyme site *Bst*1107I, ... GTA TAC ..., in the plasmid pET-28a(+) was mutated to G by the method of Deng and Nickoloff [30] using the

primers 5'-GAT AGC GGA GTG TGT ACT GGC TTA AC-3' and 5'-CAA TTC CCC TCC AGA AAT AAT TTT G-3'. Full-length human CFTR cDNA was used as a template for amplification of the base pairs corresponding to amino acids Gly-404–Lys-830 (exons 9–13), which were then inserted into the pET-28a(+) plasmid using *Nde*I and *Xho*I. Insertion was in frame with the 5' coding region of six histidines (histidine tag or ht) flanked by an optimized thrombin site (LVPRG). To obtain ht-NBF1 + R mutants in the Walker A region (K464H and K464A), cDNA cassettes were exchanged between *Bam*HI and *Bst*1107I sites with homologous cDNA cassettes excised from the mutant pMALCR1 MBP-NBF1 plasmids, constructed previously in our laboratory [31]. Both *Bam*HI and *Bst*1107I are single restriction sites that generate a region of NBF1 from Gly-438 to Val-562. This region includes the Walker A motif. For the three plasmid constructs described above, the sequence was verified in the Johns Hopkins University Synthesis and Sequencing Facility.

Overexpression of the recombinant ht-NBF1 + R (Gly-404–Lys-830) 'wild-type' and mutant proteins (K464H or K464A)

Escherichia coli BL21 (DE3) strain, freshly transformed with the 'wild-type' (i.e. recombinant) and mutant ht-NBF1 + R plasmids, were grown in 10 ml of LB medium containing 50 μ g/ml kanamycin. After growth for 3 h at 37 °C with shaking, 0.3 mM IPTG was added and allowed to induce protein synthesis for 2.5 h. Cultures expressing recombinant ht-NBF1 + R 'wild-type' and mutant proteins were performed in parallel. The induced *E. coli* were sedimented by centrifugation at 10000 g in a Sorvall GSA rotor, resuspended in Laemmli sample buffer [32], and analysed by SDS/PAGE [33] using 10% polyacrylamide gels.

For preparative cultures, 250 ml of LB medium was inoculated with 0.25 ml of the stock culture noted above, and the kanamycin concentration adjusted to 50 μ g/ml. Induction with 0.3 mM IPTG was performed when the attenuation at 660 nm reached 0.9. Growth was then continued for 1.5 h at 37 °C, after which the cultures were cooled to 4 °C and sedimented for 10 min at 6000 rev./min, also at 4 °C in a Sorvall GSA rotor. Cells were then briefly washed twice with 25 ml of Tris-buffered saline (TBS) 1 $\times$ buffer to remove bacterial waste. Cells were then sedimented for 10 min at 6000 rev./min in the same rotor at 4 °C after which the resultant pellets were stored at –20 °C.

Purification of the recombinant 'wild-type' and mutant ht-NBF1 + R proteins

Initially, experiments were carried out in which the above *E. coli* cells were lysed under non-denaturing conditions and subjected to centrifugation at high speed to obtain soluble and pellet fractions. These experiments showed that almost all protein sedimented with the pellet. For this reason, the following protocol was used to solubilize and purify the insoluble ht-NBF1 + R proteins ('wild type' and mutants) from the whole cell pellets. A volume of 5 ml of a solution containing 6 M guanidine hydrochloride, 5 mM imidazole, 1 mM dithiothreitol (fresh) and 50 mM Tris/HCl, pH 7.5, was added to dissolve an amount of the pellet corresponding to 25 ml of the original 250 ml culture. The mixture was allowed to incubate for 10 min at 37 °C with shaking, and then for 2 h at 4 °C without shaking but intermittently capping and reversing the tubes three different times. After incubation for 10 min on ice, followed by 10 min at 37 °C, the solution was stirred slowly with a magnetic stirrer overnight at 4 °C. Insoluble DNA and membrane debris were eliminated by centrifugation in a corex tube at 10000 rev./min in a Sorvall GSA rotor. The total denatured protein suspension was then

sedimented again at 10000 rev./min for 30 min at 4 °C and the supernatant applied to a 25 ml gravity-flow column containing Ni-NTA superflow equilibrated with a solution containing 50 mM Tris/HCl, pH 7.5, and 6 M guanidine hydrochloride. The column was washed with 10 column vol. of a buffer containing the same solution plus 5 mM imidazole. At this point, protein cannot be detected in the eluate by the Bradford Coomassie dye-binding assay [34]. Subsequently, by increasing the imidazole concentration in the buffer to 10 mM, non-specifically bound protein was eluted in 5 ml fractions (2 column vol.). Finally, the specifically retained 'wild-type' and mutant ht-NBF1 + R proteins were eluted in 2 ml fractions (1 column vol.) by increasing the concentration of imidazole in the buffer to 200 mM. The protein was stored in this buffer at -22 °C until use. The final yield was approx. 56 mg of 'wild-type' or mutant ht-NBF1 + R/l of starting bacterial cell culture.

Renaturation of the purified 'wild-type' and mutant ht-NBF1 + R proteins

Prior to ATPase assays, 50 μ l (0.125–2.0 mg/ml) of the pure 'wild-type' and mutant ht-NBF1 + R proteins in the denaturing buffer containing 6 M guanidine hydrochloride, 200 mM imidazole, 1 mM dithiothreitol and 50 mM Tris/HCl, pH 7.5, was diluted to 2 ml (40-fold) at 25 °C with a renaturation buffer containing 10% (v/v) glycerol, 300 mM NaCl, 1% Triton X-100, 1 mM glutathione and 50 mM Tris/HCl, pH 7.5. The mixture, which remained clear and showed no signs of precipitation or turbidity, was allowed to sit for 1 min and then assayed for ATPase activity using the two different assay procedures described below. In all cases, the duration of the assay was no more than 3 h, and usually 30 min or less as the renatured 'wild-type' and mutant NBF1 + R proteins had a tendency to become labile.

Phosphorylation of the purified 'wild-type' ht-NBF1 + R protein with protein kinase A

Purified 'wild-type' ht-NBF1 + R (20 μ l, 0.23 mg/ml) was placed in 1 ml of renaturation buffer (above) that had been supplemented with 250 μ M ATP, 10 mM MgCl₂ and 5 units of protein kinase A catalytic subunit. The mixture was then incubated for 10 min at 37 °C and phosphorylation monitored as a band shift relative to untreated 'wild-type' ht-NBF1 + R as described by Neville et al. [13].

Immunoblotting using a monoclonal antibody (MATG 1061) cross-reactive with CFTR

Following SDS/PAGE, proteins were prepared for Western blot immunodetection by electrophoretic transfer overnight at 4 °C on to PVDF membranes using a buffer containing 10 mM Caps, pH 9.5, and 10% methanol. Then non-specific binding sites on the membranes were blocked by prior incubation for 1 h at 25 °C with 2.5% non-fat milk in an NaCl/Tris/HCl solution. The membranes were then incubated for 1 h in TBS + 0.25% Tween 20 with the anti-CFTR monoclonal antibody MATG 1061 (1/5000 dilution) exhibiting specificity for NBF1. Finally, detection was effected by addition, also in 2% non-fat milk in TBS + 0.25% Tween 20, of a secondary monoclonal antibody (anti-mouse IgG coupled with horseradish peroxidase), incubation for 1 h at 25 °C and enhanced chemiluminescence.

Colorimetric assay for monitoring ATP hydrolysis

This assay is based upon using phosphomolybdate to complex the released P_i, and then using the dye Malachite Green to

intensify the colour [35]. The reaction was carried out at 37 °C in renaturation buffer containing the amounts of ATP, 'wild-type' or mutant ht-NBF1 + R, and divalent cation(s) at the concentrations indicated in the Figure legends. At the times indicated, the reaction was stopped by lowering the temperature to 4 °C. Then, 75 μ l of the reaction mixture was added to 75 μ l of ammonium molybdate tetrahydrate (7.2 g/l in 0.7 M HCl), followed by 150 μ l of Malachite Green [0.15 g/l in 0.7 M HCl and 0.1% (v/v) Triton X-100]. The absorbance of a 225 μ l aliquot of the resulting green solution in an ELISA plate reader was then determined at 630 nm in a Perkin Elmer spectrophotometer.

Radioactive assay for monitoring ATP hydrolysis

This assay, developed by Reynafarje and Pedersen [36], monitors the release of phosphate. The reaction was carried out in the presence of [γ -³²P]ATP (1.4 $\times$ 10⁶ c.p.m.) and divalent cation exactly as described in the Figure legends. At the indicated times, the reaction was quenched by the rapid addition of 150 μ l of a suspension containing 140 g/l activated charcoal (acid-washed). This binds any remaining [³²P]ATP. The mixture was then suspended by reverse pipetting and placed on a Centricon-100 filtration device that had been preloaded with 300 μ l of the same charcoal solution. After centrifugation for 5 min at 4000 rev./min in an Eppendorf microcentrifuge, the filtrate (100 μ l), containing ³²P released in the ATPase reaction, was placed in a 20 ml scintillation vial filled with 10 ml of liquid scintillation fluid (Budget Solve) and assessed for radioactivity using an LS 6000 IC Beckman scintillation counter.

SDS/PAGE and protein determination

The protocol described in Sambrook et al. [33] was followed. Electrophoresis was carried out in 10% polyacrylamide, and the gels stained either with Coomassie dye or the Silver Stain-Plus system of Bio-Rad.

Protein concentration was determined using Bio-Rad's adaptation of the Bradford Coomassie dye-binding method [34] using BSA as a standard.

RESULTS

Strategy and data acquisition

The naturally combined domains, NBF1 + R, reside in the central region of the 1480-amino-acid CFTR protein (Figure 1A). Considering that the major objective of the work described here was to obtain and study 'wild-type' NBF1 + R as a functionally active ATPase, the following strategy was followed. First, we decided to overexpress in *E. coli* amino acid residues 404–830 of CFTR, as they include the complete 'wild-type' NBF1 + R protein (Figure 1B) encoded by a complete set of exons [3,13,37]. Second, we chose the pET-28a(+) vector for the overexpression of 'wild-type' and mutant NBF1 + R proteins in *E. coli* because of this vector's established performance in the overexpression of many different proteins, including an earlier extended version of 'wild-type' NBF1 + R capable of binding the nucleotide analogues trinitrophenyl ATP and trinitrophenyl ADP [14]. Third, although we chose to use a short, N-terminally localized His tag to facilitate purification, we chose not to use a fusion protein, such as glutathione S-transferase or maltose-binding protein, in order to simplify interpretations of any positive results that might be obtained. Finally, as the capacity of 'wild-type' NBF1 + R to catalyse the hydrolysis of ATP would be used as the major criterion to ensure that proper folding had occurred, two sensitive assays were used that monitor ATPase

Figure 1 Overview of CFTR and purification and characterization of the NBF1 + R segment

(A) Schematic view of human CFTR depicting the five domains. The two fused domains NBF1 + R, the subject of this study, are indicated in grey in the central region of the 1480-amino acid polypeptide chain. Flanking on the left is TM1 and flanking on the right are TM2 and NBF2 [57]. (B) Sequence of the recombinant 'wild-type' ht-NBF1 + R region of human CFTR used in the studies reported here. Residues from Gly-404 to Lys-830 were selected for study for reasons described in the text. Attached to the N-terminal glycine (404) is a six-histidine tag together with a thrombin cleavage site (LVPR*G), not cleaved in this study. The Walker A motif is underlined, and Lys-464 within this motif is shown in bold to denote the site of mutations (K464H, K464A) made in this study. Also depicted in bold is the position of Phe-508, which when deleted causes about 70% of all cases of CF. (C) Expression in *E. coli* of the 'wild-type' ht-NBF1 + R protein and mutant forms K464H and K464A. The proteins were overexpressed in the *E. coli* BL21 (DE3) strain exactly as described in the Materials and methods section. Then, aliquots (10 μ l) were loaded on to SDS/PAGE gels (10%) and, after electrophoresis, stained with Coomassie Brilliant Blue. Lane 1, molecular-mass markers including, from top to bottom, myosin (220 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Lanes 2, 4 and 6, *E. coli* cells transformed with plasmids containing, respectively, cDNA encoding 'wild-type' ht-NBF1 + R, mutant-form K464H and mutant-form K464A prior to induction with IPTG; lanes 3, 5 and 7, as for lanes 2, 4 and 6 respectively but after induction with 0.3 mM IPTG. (D) SDS/PAGE pattern obtained at different stages of purification of the 'wild-type' ht-NBF1 + R protein and mutant forms K464H and K464A. *E. coli* cells containing the overexpressed proteins were treated with a 6 M guanidine hydrochloride-containing solution, and subjected to centrifugation steps exactly as described in the Materials and methods section. The resultant supernatants were then subjected to purification on Ni-NTA superflow columns. SDS/PAGE (10%) was used to analyse 10 μ l aliquots at each step as follows: lane 1, molecular-mass markers as defined in (C); lanes 2–4, supernatant from guanidine hydrochloride-extracted *E. coli* overexpressing the 'wild-type' ht-NBF1 + R protein and its mutant forms K464A and K464H respectively; lanes 5–7, flow-through fractions of the same extracts after their application to a Ni-NTA column (note that the $\approx$ 50 kDa proteins, i.e. the 'wild-type' ht-NBF1 + R protein and its mutant forms, were retained); lanes 8–10, eluates derived from Ni-NTA columns containing, respectively, the 'wild-type' ht-NBF1 + R protein and its K464A and K464H mutant forms after including 10 mM imidazole in the elution buffer; lanes 11–13, eluates derived from the same Ni-NTA columns after adding 200 mM imidazole to the elution buffer. Nearly homogeneous proteins were observed

activity directly [35,36]. Assays that involved 'coupling enzymes' were avoided to simplify interpretation of kinetic data and the effects of activators and inhibitors. Data presented below are generally representative of those obtained on three different 'wild-type' or mutant ht-NBF1 + R preparations. All ATPase assays were carried out in duplicate or triplicate within any given experiment and reproduced via one or more additional experiments.

Two additional criteria were used to ensure that proper folding of the ht-NBF1 + R protein had occurred. These included the appearance of a clear solution upon addition of the renaturation buffer (see the Materials and methods section), and the observance of a band shift in SDS/PAGE gels following treatment of the protein in the presence of protein kinase A and ATP to induce phosphorylation [13].

***E. coli* transformed with the pET-28a(+) expression plasmid encoding NBF1 + R residues 404–830, and a short His-tagged N-terminal region, markedly overproduce both 'wild-type' and mutant forms (K464H and K464A) of the protein**

Results presented in Figure 1(C) summarize SDS/PAGE patterns of *E. coli* cells harbouring the 'wild-type' and mutant ht-NBF1 + R-containing plasmids before and after induction with IPTG. It will be noted that in Figure 1(C), lane 3 ('wild type' with IPTG), there is an extra dark-staining band relative to lane 2 ('wild type' without IPTG). The same applies to lane 5 (mutant K464H with IPTG) versus lane 4 (mutant K464H without IPTG), and to lane 7 (mutant K464A with IPTG) versus lane 6 (mutant K464A without IPTG). In each case the extra dark-staining band is about 50–51 kDa, very near the predicted size (50.2 kDa) of ht-NBF1 + R, and identified as such in subsequent experiments. Optimal expression levels were obtained in all cases at 37 °C with 0.3 mM IPTG and a 1.5 h growth time after its addition. Significant degradation of the protein resulted after this time. Overall, it can be stated that in *E. coli* the relative expression levels of the 'wild-type' and mutant ht-NBF1 + R proteins are nearly identical with no sign of degradation under the conditions used.

The ht-NBF1 + R 'wild-type' protein and its mutant forms can be purified to apparent homogeneity on a Ni-NTA column and show cross-reactivity with a monoclonal antibody to CFTR exhibiting specificity for NBF1

As the overexpressed 'wild-type' and mutant (K464H and K464A) proteins were tagged with six histidines at their N-terminus (see the Materials and methods section and Figure 1B), they were amenable to purification on a column containing bound Ni²⁺. For this reason, the wild-type and mutant *E. coli* cell pellets derived from the overexpression experiments described above were treated with a buffered solution containing the denaturant guanidine hydrochloride (6 M), and after centri-

fugation to remove insoluble membrane debris and DNA, the supernatant was applied in each case to an Ni-NTA column. Results presented in Figure 1(D) show that, although numerous proteins in the guanidine hydrochloride-solubilized 'wild-type' and mutant fractions of *E. coli* passed through the NTA column (Figure 1D, lanes 5–7), the overexpressed protein exhibiting a molecular mass near that of 'wild-type' ht-NBF1 + R (50.2 kDa) remained bound in each case (Figure 1D, lanes 2–4 compared with lanes 5–7). Very little of the 'wild-type' and mutant proteins were removed when 10 mM imidazole was included in the eluting buffer (lanes 8–10), but inclusion of 200 mM imidazole removed the 'wild-type' and mutant proteins in nearly homogeneous form (lanes 11–13). Data presented in Figure 1(E) show that the purified 'wild-type' ht-NBF1 + R protein (lane 1) and its K464A mutant (lane 2) cross-reacted with a monoclonal antibody to CFTR exhibiting specificity for NBF1. As expected, the antibody also cross-reacted with the positive control, an Intein-NBF1 fusion protein (lane 3), and failed to cross-react with the negative control, bovine albumin (lane 4). The yield of the purified, denatured 'wild-type' and mutant ht-NBF1 + R proteins was quite high (56 mg/l of starting bacterial culture), providing generous amounts of starting material for the renaturation and ATPase studies described below.

The purified 'wild-type' and mutant ht-NBF1 + R proteins can be renatured at 25 °C upon rapid dilution with a buffered solution containing glycerol

In order to identify conditions that allow folding of the denatured 'wild-type' ht-NBF1 + R protein into a functionally active ATPase, we examined carefully those conditions that had been used earlier to renature other recombinant preparations of this protein sufficiently to bind ATP analogues [13,14]. In one case, rapid dilution at 4 °C with an acetate-buffered solution was used [13]; in a second case dialysis was carried out at 4 °C against phosphate-buffered solutions, pH 7.5, containing gradually decreasing concentrations of urea [14]; and finally, in a third case, dialysis was carried out at 4 °C in a Tris/HCl-buffered solution, pH 7.5, containing arginine [14]. Noting that the common denominator among these three different renaturation conditions was low temperature, this suggested that perhaps this was a barrier to the earlier preparations [13,14], folding completely into functional ATPases. With this thought in mind and after much trial and error, we finally found that rapidly diluting (1/40) the 'wild-type' denatured ht-NBF1 + R protein at 25 °C into a buffered solution containing 10% (v/v) glycerol, 300 mM NaCl, 1% Triton X-100, 1 mM glutathione and 50 mM Tris/HCl, pH 7.5, resulted in an active ATPase as described in the next section. We also showed (Figure 1F) that when protein kinase A was included in this renaturation buffer together with ATP and MgCl₂, the 'wild-type' ht-NBF1 + R protein underwent a band shift upon SDS/PAGE characteristic of the phosphorylated protein [13]. This treatment, although of general interest, was not

on the SDS/PAGE gels for the 'wild-type' ht-NBF1 + R protein (lane 11) and its mutant forms K464A (lane 12) and K464H (lane 13). (E) Cross-reactivity of the purified 'wild-type' ht-NBF1 + R protein and mutant form K464A with an anti-CFTR antibody (MATG 1061) with specificity for the NBF1 domain. SDS/PAGE gels containing the purified proteins were transferred to PVDF membranes and then analysed for cross-reactivity with an NBF1 antibody (MATG 1061) exactly as described in the Materials and methods section. Lanes 1 and 2, purified 'wild-type' ht-NBF1 + R protein (0.5 µg) and its K464A mutant (0.5 µg), respectively. Lanes 3 and 4, positive and negative controls, respectively. The positive control was an Intein-NBF1 fusion protein (5 µg, ≈ 86 kDa, NBF1 residues Trp-401–Lys-684) prepared using recent Intein technology [58]. The negative control was BSA (0.5 µg, ≈ 66 kDa). (F) Band shift of the 'wild-type' ht-NBF1 + R protein after treatment under renaturation conditions with protein kinase A and ATP. The purified 'wild-type' ht-NBF1 + R protein was treated under renaturation conditions with and without protein kinase A and ATP exactly as described in the Materials and methods section. Immediately thereafter, samples were subjected to SDS/PAGE (10% gels) and then subjected to silver staining also as described in the Materials and methods section. Lane 1, molecular-mass markers (identical to C but with trypsin inhibitor, 20.1 kDa, included); lane 2, control with no protein added; lanes 3 and 4, 'wild-type' ht-NBF1 + R protein (25 ng) renatured in the absence and presence, respectively, of protein kinase A and ATP.

Figure 2 ATPase activity of the ht-NBF1 + R protein and inhibitory effects of mutations and vanadate

(A) Demonstration that the method for determining P_i using phosphomolybdate and Malachite Green results in a linear absorbance (630 nm) versus $[P_i]$ plot and is highly sensitive. The method as used here detects $[P_i]$ of less than 10 μM . (B) ATPase activity versus $[ATP]$ plot for the purified renatured 'wild-type' ht-NBF1 + R protein and mutant forms K464H and K464A. ATPase activity was assayed by the colorimetric method using phosphomolybdate and Malachite Green as described in the Materials and methods section. The assay contained, in 75 μl of renaturation buffer, ATP concentrations of 0 – 100 μM , 5 mM $MgCl_2$ and 0.47 μg of either the 'wild-type' ht-NBF1 + R protein (WT) or its mutant forms K464H and K464A, pH 7.5 . The assay was carried out for 30 min at 37 $^{\circ}C$ and then subjected to P_i determination. (C) Calculation of the apparent K_m and V_{max} for the ATPase activity of the 'wild-type' ht-NBF1 + R protein from the data in (B). An Eadie–Hofstee plot [38,39] of v versus v/S was made. V_{max} was obtained from the y axis and K_m from the slope. (D) Relative ATPase activities obtained for the 'wild-type' NBF1 + R Protein (WT) and mutant forms K464H and K464A using the $[^{32}P]Pi$ release assay. The assay was carried out in 200 μl of renaturation buffer, pH 7.5 , containing 3.7 μg of the 'wild-type' ht-NBF1 + R protein or its mutant forms (K464H, or K464A), and supplemented with 250 μM $[\gamma\text{-}^{32}P]ATP$ (1.4×10^6 c.p.m.) and 1 mM divalent cation. After 20 min incubation at 37 $^{\circ}C$ the $[^{32}P]Pi$ released was quantified as described in the Materials and methods section. (E) Inhibition of the ATPase activity of the 'wild-type' ht-NBF1 + R protein by vanadate. The assay was carried out in 225 μl of renaturation buffer, pH 7.5 , containing 20 μg of 'wild-type' ht-NBF1 + R protein and supplemented with 0 – 150 μM $[\gamma\text{-}^{32}P]ATP$ (1.4×10^6 c.p.m.), 1.1 mM $MgCl_2$ and, where indicated, 220 μM orthovanadate (V_i) that had been prior-treated to remove polymeric species [29]. After 180 min incubation at 37 $^{\circ}C$, the $[^{32}P]Pi$ released was quantified as described in the Materials and methods section. The percentage of ATPase activity remaining was based on the total activity obtained at 150 μM ATP in the absence of vanadate.

Figure 3 Capacity of divalent cations to activate or inhibit the ATPase activity of the ht-NBF1 + R protein

(A) Relative capacities of Co^{2+} and Mg^{2+} to serve as divalent cation activators of the ATPase activity catalysed by the 'wild-type' ht-NBF1 + R protein. The assay was carried out at 37 °C in 300 μl of renaturation buffer, pH 7.5, containing 0.75 μg of 'wild-type' ht-NBF1 + R protein and supplemented with 250 μM ATP, and 2 mM MgCl_2 or CoCl_2 . At the times indicated, a 37.5 μl aliquot was removed and analysed for P_i by the colorimetric procedure using phosphomolybdate and Malachite Green (see the Materials and methods section). In a separate experiment carried out in an identical manner MgCl_2 was omitted. All experiments also included a control in which the 'wild-type' ht-NBF1 + R protein was omitted. (B) Relative capacities of five different divalent cations to activate the ATPase activity of the 'wild-type' ht-NBF1 + R protein. The assay was carried out at 37 °C in 300 μl of renaturation buffer, pH 7.5, containing 1.9 μg of 'wild-type' ht-NBF1 + R protein and supplemented with 250 μM ATP and 5 mM of each divalent cation as indicated. After 30 min, a 75 μl aliquot was removed and analysed for P_i by the colorimetric procedure using phosphomolybdate and Malachite Green (see the Materials and methods section). In separate experiments controls were run where either divalent cation or the 'wild-type' ht-NBF1 + R protein was omitted from the assay. Values are presented as means $\pm$ S.D. (C) The inhibitory effect of Cd^{2+} on the Co^{2+} -activated ATPase activity of the 'wild-type' ht-NBF1 + R protein. The assay was carried out at 37 °C in 200 μl of renaturation buffer, pH 7.5, containing 3.7 μg of 'wild-type' ht-NBF1 + R protein and supplemented with 250 μM [γ - ^{32}P]ATP (1.4×10^6 c.p.m.). Where indicated, divalent cations were added to give a final concentration for each of 1 mM. After a 20 min incubation the reaction was analysed for $^{32}\text{P}_i$ released as described in the Materials and methods section. The activity is expressed relative to that observed in the presence of cobalt alone.

required for the 'wild-type' ht-NBF1 + R protein to catalyse ATP hydrolysis in the studies described below and for this reason was omitted.

Most preparations of the renatured nt-NBF1 + R protein were stable for about 30 min although some did retain stability for more than 1 h. When the protein became labile this was readily

Figure 4 Inhibitory effect of Cd^{2+} on the ATPase activity of the ht-NBF1 + R protein and co-ordination geometry and ionic radius of each divalent cation tested in this study

(A) Inhibitory effect of Cd^{2+} on the Co^{2+} -activated ATPase activity of the 'wild-type' ht-NBF1 + R protein at different concentrations of ATP. The assay was carried out at 37 °C in 75 μl of renaturation buffer, pH 7.5, containing 0.47 μg of 'wild-type' ht-NBF1 + R protein supplemented with ATP (0–100 μM) and, where indicated, 5 mM CoCl_2 and 1 mM CdCl_2 . After 20 min the reaction medium was analysed for released phosphate by the colorimetric assay employing phosphomolybdate and Malachite Green (see the Materials and methods section). The activity is expressed relative to that observed in the presence of Co^{2+} and 100 μM ATP. (B) Inhibitory effect of Cd^{2+} on the Mg^{2+} -activated ATPase activity of the 'wild-type' ht-NBF1 + R protein at different concentrations of ATP. All conditions were identical to (A) except that CoCl_2 was replaced with MgCl_2 . Here, the activity is expressed also relative to that observed in the presence of Co^{2+} and 100 μM ATP. (C) Preferred co-ordination geometry and ionic radius (IR) of each divalent cation examined in this study for its effect on the ATPase activity of 'wild-type' ht-NBF1 + R. Co^{2+} , Mn^{2+} and Mg^{2+} can form octahedral square bipyramidal geometries in interacting with ligands (L) at the active sites of enzymes, whereas Cd^{2+} can form either a square pyramidal or a tetrahedral geometry [54,55]. The ability of these cations to activate the ATPase activity of 'wild-type' ht-NBF1 + R is inversely related to their ionic radius, i.e. in the order $\text{Co}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+}$, with Cd^{2+} having no activating capacity; rather, it has the capacity to compete with the activators, thus inhibiting ATPase activity of the 'wild-type' ht-NBF1 + R.

detected by a milky appearance upon direct visualization, or by an unexpected increase in absorbance at 340 nm (light scattering) concomitant with a rapid decline in ATPase activity.

The purified, renatured 'wild-type' ht-NBF1 + R protein readily catalyses the hydrolysis of ATP at a single high-affinity site by a mechanism that is disrupted both by mutations in the Walker A region and by the transition-state analogue orthovanadate

To assess whether the purified, renatured 'wild-type' ht-NBF1 + R protein has the capacity to catalyse the hydrolysis of ATP, we used two different assays for monitoring ATP hydrolysis, both of which monitor directly the release of P_i (see the Materials and methods section). In one of these assays the released P_i is complexed first with phosphomolybdate, plus Malachite Green, and monitored by measuring the absorbance at 630 nm, while in the other assay the [32 P]Pi released from [γ - 32 P]ATP is rapidly separated from the latter via a charcoal-containing filter and its radioactivity assessed. Results presented in Figure 2(A) show that the phosphomolybdate/Malachite Green pair has a detection level that extends below 5 μ M P_i . Using this assay to monitor P_i release, we showed first that P_i was released from ATP in a linear manner for at least 30 min when the 'wild-type' ht-NBF1 + R protein was added to initiate the reaction. Therefore, working within this time range, we first collected sufficient data at different ATP concentrations to obtain a plot of velocity versus substrate concentration (Figure 2B). These data revealed typical Michaelis–Menten kinetics for the 'wild-type' ht-NBF1 + R ATPase (Figure 2B), which upon further analysis via an Eadie–Hofstee plot [38,39] were consistent with ATP hydrolysis at a single catalytic site with a K_m of 8.7 μ M (Figure 2C). The maximal velocity (V_{max}) was 135 nmol/min per mg [catalytic-centre activity (turnover number), 6.7 min $^{-1}$]. Subsequent studies carried out with ht-NBF1 + R proteins with mutations in the Walker A consensus region (K464H and K464A) showed that the V_{max} was reduced about 50 % in each case (Figure 2B). A similar result was obtained using the assay in which [32 P]Pi release from [γ - 32 P]ATP was monitored (Figure 2D), although here the inhibitory effect of the mutations on the 'wild-type' ht-NBF1 + R ATPase activity was somewhat greater. Finally, it can be seen in Figure 2(E) that orthovanadate, known to mimic transition states in several ATPase catalysed reactions [29,40,41], markedly inhibits the ATPase activity of 'wild-type' ht-NBF1 + R. Here also, the assay monitoring [32 P]Pi release from [γ - 32 P]ATP was used.

The ATPase activity catalysed by the 'wild-type' ht-NBF1 + R protein exhibits a requirement for divalent cations similar to that observed for mitochondrial F_1 -ATPase

The few studies that have been conducted to date on the ATPase activity of isolated CFTR and its soluble domains have almost always employed Mg^{2+} as a divalent cation activator (reviewed in [9,42]). This has also been the case for most studies where the effects of ATP or its analogues have been studied on CFTR's chloride channel activity in intact cells or in patch-clamp studies (reviewed in [6,7]). However, as we have shown previously [28], NBF1 of CFTR has significant homology and similarity in its amino acid composition to the β -subunit (catalytic subunit) of mitochondrial ATP synthase's F_1 moiety, the ATPase activity of which is activated as well by Co^{2+} as by Mg^{2+} [43]. For this reason, we tested Co^{2+} for its capacity, relative to that of Mg^{2+} , to serve as a divalent cation activator of the ATPase activity of 'wild-type' ht-NBF1 + R. The results presented in Figure 3(A) show that 2 mM Co^{2+} , when examined over a time course of

30 min, was in fact superior to Mg^{2+} in activating the ATPase activity of 'wild-type' ht-NBF1 + R. In both cases, the amount of divalent cation present (2 mM) was more than sufficient to complex the amount of ATP (250 μ M) present in the assay. Although the specific ATPase activity of different 'wild-type' ht-NBF1 + R preparations varied somewhat, it was found consistently that Co^{2+} was superior to Mg^{2+} in activating this activity.

As shown in Figure 3(B) we extended the observation with Co^{2+} and Mg^{2+} to include several other divalent metal cations known to activate and/or be transported by other known ATPases [44–46]. Here it can be seen that Co^{2+} was a significantly better activator of the 'wild-type' ht-NBF1 + R ATPase activity than its close relative Mn^{2+} , which in turn was a better activator than Mg^{2+} . Interestingly, Ca^{2+} and Cd^{2+} had no capacity to activate the ATPase activity of 'wild-type' ht-NBF1 + R. Also, in this experiment where the divalent cation concentration was 5 mM rather than the 2 mM used in the experiment summarized in Figure 3(A), it can be seen that the ATPase activation increment of Co^{2+} over Mg^{2+} was even more pronounced.

The divalent cation Cd^{2+} is a potent inhibitor of the divalent-metal-cation-activated ATPase activity of 'wild-type' ht-NBF1 + R

As Cd^{2+} is distantly related to the other divalent metals tested, and has no ATPase-activating capacity, we rationalized that it might exhibit inhibitory properties. Data presented in Figure 3(C) confirm that this is the case as it can be seen that when Co^{2+} , the best activator, is placed in the presence of Cd^{2+} , both at 1 mM, the activating capacity of Co^{2+} is almost completely prevented. In experiments not presented here, increasing the Co^{2+} concentration to 5 or 10 mM relieved the inhibition by Cd^{2+} about 40 and 80 %, respectively, suggesting that Co^{2+} and Cd^{2+} may be competing for the same site. Results presented in Figure 4(A) show that the capacity of Cd^{2+} to inhibit the Co^{2+} -activated ATPase activity of 'wild-type' ht-NBF1 + R was retained even at concentrations of ATP (100 μ M) that were > 10 times higher than the K_m (8.7 μ M). Moreover, the capacity of Cd^{2+} to inhibit the ATPase activity of 'wild-type' ht-NBF1 + R was not specific for Co^{2+} . Thus, as shown in Figure 4(B), Cd^{2+} is also a potent inhibitor of the Mg^{2+} -activated ATPase activity.

In summary, it seems clear that the ATPase active site of NBF1 within the purified 'wild-type' ht-NBF1 + R protein can accommodate a variety of divalent cations that range from excellent activators of ATPase activity to potent inhibitors.

DISCUSSION

The novel set of studies described in this report focused on CFTR's central region, the NBF1 + R combined domains, i.e. residues Gly-404–Lys-830 encoded by exons 9–13. Significantly, this region holds a number of key secrets related to both the function and regulation of CFTR, and to why so many mutations located here, in particular within NBF1, cause CF. Specifically, in the experiments described, we have been able to prepare large quantities of the 'wild-type' and mutant ht-NBF1 + R proteins in purified denatured form and then rapidly refold small quantities. Most importantly, we have demonstrated that the refolded 'wild-type' protein exhibits significant ATPase activity characterized by a high apparent affinity for ATP and a marked sensitivity to vanadate. Finally, we have identified several divalent cations (Co^{2+} , Mn^{2+} and Mg^{2+}) that activate the ATPase activity, and have identified Cd^{2+} as an effective inhibitor. These findings merit further discussion as presented below.

Generally, no major problems were encountered in overexpressing the 'wild-type' and mutant ht-NBF1 + R proteins in bacteria. This went according to plan as described in detail in the text with no obvious signs of degradation during purification. Also, the protein was readily purified to apparent homogeneity in denatured form on a Ni-NTA superflow column, resulting in abundant quantities of 'wild-type' and mutant ht-NBF1 + R proteins (≈ 56 mg/l of starting bacterial culture). Refolding the 'wild-type' ht-NBF1 + R protein into a form exhibiting the capacity to hydrolyse ATP was more time consuming, as initially much effort was invested employing procedures used previously that had been successful in folding denatured NBF1 + R sufficiently to bind nucleotide analogues of ATP or ADP [13,14]. However, these methods, all of which employed low temperature (4°C), were unsuccessful in our hands in obtaining a 'wild-type' ht-NBF1 + R protein with the capacity to hydrolyse ATP. After switching the temperature to 25°C , and using a refolding cocktail containing glycerol, described in the Materials and methods section, we were able to show that the denatured 'wild-type' ht-NBF1 + R protein can be folded under dilute conditions into a catalytically active enzyme with the capacity to hydrolyse ATP. As it is known that hydrophobic forces that stabilize proteins are weakened as temperature is lowered [47], it seems likely that such interactions contribute significantly to the stability of the final folded quaternary structure of 'wild-type' ht-NBF1 + R. One of these critical stabilizing interactions may involve Phe-508, which is located in a hydrophobic region of CFTR [3] and, when deleted, causes most cases of CF [1,2].

It should be noted that the final folded, 'wild-type' ht-NBF1 + R protein exhibiting an ATPase activity contains no additional cargo, i.e. neither another CFTR domain nor a fusion protein. Thus the 'wild-type' ht-NBF1 + R is able to fold essentially alone into a catalytically active protein. As expected, the maximal turnover number obtained (6.7 min^{-1}) for the ATPase activity of the 'wild-type' ht-NBF1 + R protein is less than that reported for intact CFTR where values range from 8.6 to 60 min^{-1} (calculated from data in the literature [48–50] and a molecular mass for CFTR of 168.1 kDa). Considering that intact CFTR has two nucleotide domains whereas the 'wild-type' ht-NBF1 + R protein studied here has only one, the turnover number of 6.7 min^{-1} seems reasonable. Finally, although data presented here may appear inconsistent with the recent proposal that both nucleotide domains of an ABC transporter must form dimers with connecting 'face-to-face' ATP-binding pockets for ATP hydrolysis to occur [51,52], we cannot exclude the possibility that 'wild-type' ht-NBF1 + R dimerizes under our experimental conditions.

The apparent K_m (ATP) of $8.7\text{ }\mu\text{M}$ reported here for the ATPase reaction catalysed by 'wild-type' ht-NBF1 + R is significantly lower than previous values [10,15,16,48,49], e.g. those reported for isolated NBF1 alone (K_m , $250\text{ }\mu\text{M}$), for fusion proteins containing NBF1 (60 and $110\text{ }\mu\text{M}$) and for intact CFTR (K_m , $\approx 1\text{--}3\text{ mM}$). In all cases, values refer to proteins that had not experienced prior phosphorylation by added protein kinases. The lower K_m (ATP) for the 'wild-type' ht-NBF1 + R protein reported here, taken together with the earlier higher reported K_m values for isolated NBF1 or fusion proteins thereof, suggest that NBF1 has the capacity to undergo conformational 'switches' between high- and low-affinity states. Finally, the much higher K_m (ATP) of $1\text{--}3\text{ mM}$ for the ATPase activity catalysed by intact CFTR than by isolated NBF1 or NBF1-containing proteins, all in the μM range, poses an interesting dilemma. Among the possible interpretations, one is that within intact isolated CFTR the affinity of NBF1 for ATP is negatively affected, perhaps by the second nucleotide-binding domain (NBF2), while a second

possibility is that the conditions necessary to maintain isolated CFTR in soluble or liposomal form [48,49] may lower the affinity of NBF1 for ATP.

Although we did not study in detail the effect of phosphorylation of the R domain on the ATPase activity of the ht-NBF1 + R protein, we did show that treatment of this protein with protein kinase A and ATP induced a band shift in SDS/PAGE gels, previously shown to be a result of phosphorylation [13]. In experiments not presented here, we found also that the treated ht-NBF1 + R protein exhibited no change in ATPase activity (J. P. Annereau, unpublished work). As phosphorylation of the R domain has been shown to decrease the K_m of CFTR for ATP [48], the combined observations would suggest that a domain other than NBF1 (most likely NBF2) is responsible for the phosphorylation-dependent K_m change observed in the complete protein.

In the final phase of work reported here, we assessed the relative capacities of several divalent metal cations to satisfy the metal-ion requirement of the ATPase activity of the 'wild-type' ht-NBF1 + R protein. Divalent metal cations are required to activate most known ATPases and can play multiple roles, e.g. aiding in the binding of ATP and interacting with the attacking water molecule [53]. Here, patterning our approach after an earlier study on the F_1 (ATPase) moiety of mitochondrial ATP synthase [43], we selected Co^{2+} , Mn^{2+} , Ca^{2+} and Cd^{2+} in addition to Mg^{2+} . The results of our study showed clearly that, under the conditions used here, the commonly used Mg^{2+} ion is not the best activator of the 'wild-type' ht-NBF1 + R ATPase activity. Rather, both Co^{2+} and Mn^{2+} are superior, whereas Ca^{2+} and Cd^{2+} are ineffective as activators. In fact, upon further study Cd^{2+} was shown to be an excellent inhibitor of both the Co^{2+} - and Mg^{2+} -induced ATPase activity catalysed by 'wild-type' ht-NBF1 + R.

These studies on the divalent metal cation specificity of 'wild-type' ht-NBF1 + R ATPase are useful as they provide clues about the nature of the active site of this protein. Thus as shown in Figure 4(C), Co^{2+} , Mn^{2+} and Mg^{2+} , unlike Cd^{2+} , can all assume an octahedral co-ordination geometry at enzyme active sites [54,55]. Moreover, the ionic radius is less for Co^{2+} than for Mn^{2+} and that of Mn^{2+} is less than Mg^{2+} [54,55], i.e. in the same order as their capacity to activate the ATPase activity of the 'wild-type' ht-NBF1 + R protein. In sharp contrast, the ionic radius of the inhibitory cation Cd^{2+} is much larger than that of the activating cations. Ca^{2+} , impotent as an activator of the ATPase activity of 'wild-type' NBF1 + R, also assumes a co-ordination geometry (decahedral pentagonal bipyramidal) distinct from the activating cations and a much larger ionic radius [54,55]. Taken together, these data indicate that the catalytic site within 'wild-type' ht-NBF1 + R may be very much like that within the F_1 moiety of the mitochondrial ATP synthase, i.e. exhibiting a preference for a relatively small divalent cation in terms of ionic radius that can assume an octahedral geometry. Further suggestive evidence for this is the finding reported here that the ATPase activity of the 'wild-type' ht-NBF1 + R protein is inhibited by vanadate, also known to be a transition-state-like inhibitor of mitochondrial F_1 [29]. However, although similar, the active sites of 'wild-type' ht-NBF1 + R and F_1 must have at least one major difference as the turnover number for F_1 -catalysed ATP hydrolysis is nearly 400 s^{-1} whereas that of 'wild-type' ht-NBF1 + R is no more than 6.7 min^{-1} (0.11 s^{-1}). Obtaining significant clues as to what this difference is must await an atomic resolution structure of either NBF1 + R or intact CFTR.

In conclusion, the results of studies described here represent another significant step forward in our quest to ultimately obtain a fully active NBF1 + R/NBF2 complex that can be subjected

to structural studies. In addition, they may be very relevant to understanding the active sites and catalytic mechanisms of other ABC transporters. For example, MRP6 (ABCC6) has been reported recently to bind ATP also in the presence of Co^{2+} , Mn^{2+} or Mg^{2+} [56]. Finally, an extension of these studies may prove useful in selecting novel, divalent-cation-based 'small-molecule' activators and inhibitors of CFTR's chloride channel function, which is believed to be directly or indirectly dependent on the ATPase activities catalysed by its nucleotide domains.

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